COMMENTARY

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Vaccine and adjuvant design for emerging viruses

Mutations, deletions, segments and signaling

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Vaccination is currently the most effective strategy to medically control viral diseases. However, developing vaccines is a long and expensive process and traditional methods, such as attenuating wild-type viruses by serial passage, may not be suitable for all viruses and may lead to vaccine safety considerations, particularly in the case of the vaccination of particular patient groups, such as the immunocompromised and the elderly. In particular, developing vaccines against emerging viral pathogens adds a further level of complexity, as they may only be administered to small groups of people or only in response to a specific event or threat, limiting our ability to study and evaluate responses. In this commentary, we discuss how novel techniques may be used to engineer a new generation of vaccine candidates as we move toward a more targeted vaccine design strategy, driven by our understanding of the mechanisms of viral pathogenesis, attenuation and the signaling events which are required to develop a lasting, protective immunity. We will also briefly discuss the potential future role of vaccine adjuvants, which could be used to bridge the gap between vaccine safety and lasting immunity from a single vaccination.

Key words: viral vaccines, adjuvants, emerging viruses, biodefense, arenaviruses, bunyaviruses, filoviruses, flaviviruses

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Introduction

The introduction of West Nile virus into the US in New York in 1999, and its subsequent rapid spread across North America perfectly illustrates the ability of viruses to rapidly emerge into new areas and threaten new populations. Emerging infections present myriad challenges for vaccine design.[1] However, historically, infections we now refer to as emerging or biothreat agents have been the targets of successful vaccines including yellow fever and, perhaps the most striking example, smallpox, which has been eradicated from the wild thanks to a coordinated worldwide vaccination campaign. A summary of vaccines against emerging viruses can be found in reference 1; an overview of the potential limitations of some of the novel vaccine development strategies discussed in this commentary is shown in **Table 1**.

A major area of virus research is the investigation of virus infection on host responses and pathogenesis, in particular, how viruses interact with the immune system, either activating particular pathways, or by inhibiting specific immune mechanisms to facilitate their replication. A more complete understanding of these interactions may provide information, which could feed into the vaccine development pipeline.

One significant field of research is that of comparing host responses to virulent, pathogenic viruses, versus a similar virus, which does not cause disease. In this way, we can begin to dissect the cellular responses that may be associated with disease pathogenesis, and which may lead to the development of a protective immune response. We have previously used comparisons between attenuated and virulent strains of a hemorrhagic arenavirus, a model of lassa fever, to define host-response pathways associated with

Table 1. Potential limitations of vaccine strategies

Strategy	Example(s)	Potential limitations
Live-attenuated virus	Yellow fever 17D; Candid#1 Junin virus	Adverse events; issues with use in immunocompromised populations; reversion to virulence
Inactivated virus	Inactivated 17D vaccine;[41] inactivated Japanese encephalitis virus vaccine	Potential requirement for multiple booster vaccinations; shorter duration of protection; risk of incomplete inactivation
Recombinant virus	VSV-based Ebola vaccine;[20] Junin: TC83 virus	Immune response limited to glycoprotein, may miss protective T-cell epitopes in other viral proteins
Virus-like particle	Lassa and Ebola vaccine candidates[29,49]	Similar to inactivated virus, although without incomplete inactivation risk; lack of nucleic acid may impact activation of cellular pathogen recognition receptors
Reassortant virus	Lassa/Mopeia reassortant[25]	Limited to multi-segment viruses; requires apathogenic virus; risk of adverse events
Engineered T-cell epitope peptide	Pan-arenavirus candidate vaccine[50]	Limited to production of cytotoxic T-cell responses

virulence or the development of protective responses.[2-6] Similar studies have also been undertaken in other virus-host systems.[7,8] By better understanding these cellular responses, we may be able to begin to design vaccines, which stimulate appropriate immune signaling pathways and lead to a lasting protective response.

In this commentary, we will present a brief review of some of the emerging technologies that are facilitating the development of the next generation of vaccines to emerging viruses and biothreat agents. We will comment on how basic science at the level of understanding of the host response can allow targeted development of vaccine candidates and potentially lead to the engineering of novel vaccine adjuvants. Using a cross-section of examples and considering some current opinions in the field of vaccinology, we will speculate on the future of biodefense vaccine development in terms of novel vaccine candidates and adjuvant strategies.

Exploiting Viral Virulence Differences

The ability of a virus to infect and cause disease in a host is dependent on the interplay between the pathogen, the host and the environment. Minor differences in any of these components can vastly affect the outcome following exposure. Examples of subtle sequence differences affecting the virulence of a virus can be seen with hemorrhagic fever viruses. *Ebola virus*, one of the better-known hemorrhagic fever viruses, has a number of different strains including Zaire (ZEBOV), Sudan (SEBOV) and Reston (REBOV).[9] ZEBOV has a mortality rate approaching 90% in those with clinical symptoms of disease. SEBOV has a mortality rate between 50 and 65%, while REBOV has never been shown to cause clinical disease in humans.[9,10] This is particularly interesting, as REBOV and SEBOV both share approximately 61% sequence identity with ZEBOV. Similar differences have been observed between strains of *Rift Valley Fever virus* (a hemorrhagic bunyavirus) and related avirulent strain Clone 13, as well as *Lassa virus* and its avirulent relative *Mopeia virus*.[11,12]

Variation between hosts also plays a major role in the virulence of pathogenic viruses. From the possession of particular HLA subtypes to favorable immunoglobulin gene rearrangements, there are a number of reasons that one individual may not develop an infection under the same conditions as someone who does. An example of such diversity of pathology is that seen following infection with West Nile virus. In the majority of cases, the disease is asymptomatic, however, some patients develop West Nile fever and a small proportion develop encephalitis.[13]

Both of the above mechanisms of variability leading to altered viral virulence can be exploited in terms of development of vaccines and therapies. Naturally occurring avirulent strains of viruses can allow for the better understanding of the virulence of the pathogenic strains. This in turn may allow for the virulent strains to be mutated in such a manner as to render the virus avirulent while maintaining immunogenicity. The ability to identify immune response correlates of pathogenicity or protection can allow for their inhibition and upregulation respectively through the administration of particular adjuvants alongside vaccination. This will promote the induction of a favorable immune response while limiting the development of unwanted side effects. In addition, if pathogenic immune elements could be inhibited during a virulent infection, the overall outcome may be able to be improved significantly.

This last statement illustrates the potential overlap between vaccine design, therapeutic development and diagnostic and prognostic biomarker discovery, and shows the nascent power of understanding which cellular responses correlate with severe disease, and which with protective responses. If these events can be defined, then can particular markers of the response be used to determine the likely course of disease as a prognostic marker? In the case of epidemics of disease where there may be large numbers of 'worried well', but limited healthcare resources, a reliable prognostic assay would be a significant clinical benefit. Also, if we know which immune events are associated with protection and how these may be inhibited in virulent infection, can we modulate these pathways with novel therapeutics which act as antivirals by assisting in the development of a protective immune response? A proof of concept has already been shown, with DNA 'thioaptamers' containing transcription factor binding sites showing modulation of the outcome in a lassa fever model system.[4,14]

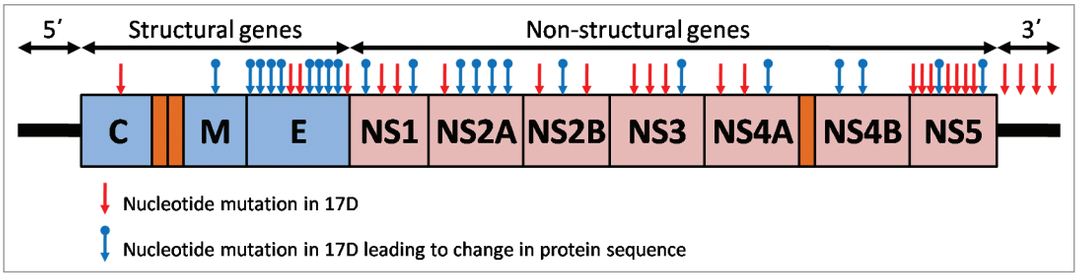

Figure 1. Nucleotide and amino acid mutations in the 17D yellow fever vaccine. The figure shows the genome-wide distribution of mutations in the live-attenuated yellow fever vaccine as a result of serial passage from the virulent Asibi strain. One additional nucleotide mutation is present in the rNS3 gene; this has been omitted from the figure for clarity. As can be seen, mutations are present across all viral genes. As may be expected, the majority of mutations in the E (envelope) proteins lead to amino acid changes, which may affect the ability of the virus to infect different cell types. Mutations in the 3' non-coding region of the genome could affect the binding capability of specific regulatory proteins.

Viral Genome Manipulation for Vaccine Development

The hemorrhagic fever-causing filoviruses and flaviviruses are comprised of a single segment of RNA. The most striking example of the development of an effective vaccine against a hemorrhagic fever virus, is that of the 17D yellow fever vaccine. The 17D vaccine was developed in the 1930s using a technique applied in the development of many virus vaccines: repeated passage leading to the development of an attenuated variant. The sequencing of the complete 17D genome revealed 48 nucleotide changes compared to the parent Asibi strain.[15] These nucleotide changes lead to 22 amino acid mutations, which are distributed throughout the genome (**Fig. 1**).

For viruses with large genomes encoding many genes, such as *Variola virus*, the etiological agent of smallpox, or the agricultural biothreat *African swine fever virus* (ASFV), entire genes or regions of the genome may be deleted, without significantly altering replication. This may provide a further means of developing live attenuated vaccines. Again, an understanding of virus-host interactions can drive the search for novel vaccine development. If we can identify virus genes specifically associated with pathogenesis, such as those that inhibit host immune responses, these can be deleted and the effect of that deletion on virus growth and fitness can be observed. In the case of ASFV, the virus *A238L* gene is responsible for inhibiting the NFκB transcription factor, a central regulator of inflammation and the innate immune response.[16,17]

However, despite the fact that this gene is conserved among ASFV isolates, deleting this gene made no difference to the ability of the virus to replicate in culture or to cause disease in domestic swine.[18] While apparently not required for pathogenesis, this gene may be required for maintaining the virus in the reservoir hosts, warthogs, bushpigs and soft ticks. Deletion of the multigene families 360 and 530, which may play a role in inhibiting the host interferon response, led to a reduction in growth in culture,[18,19] illustrating the importance of virus modulation of immune responses in determining the severity of infection.

A strategy showing significant promise is the production of recombinant viruses expressing the envelope proteins of the vaccine target virus in the backbone of a non-pathogenic virus. One particular vaccine candidate showing promise is a recombinant vesicular stomatitis virus, which encodes either the EBOV or *Marburg virus* (MARV) glycoprotein. A single dose of this vaccine was able to induce a protective response and protect from disease following challenge with virulent virus.[20] This vaccine was also shown to be protective if given post-exposure and against challenge via the aerosol route.[21,22]

At the level of viral interaction with the cellular response, inhibition of the immune response-regulating transcription factor IRF-3 is critical in the development of pathogenesis of EBOV. The inhibition of IRF-3 activity is performed by the viral VP35 protein. A single amino acid mutation in this protein is capable of disrupting this effect and renders the virus completely apathogenic in mice.[23] Whole genome expression profiling indicated that this one mutation led to a striking effect on multiple immune response pathways.[24] Findings such as this indicate the potential power of how understanding virus-host interactions can lead to the targeting of specific mutations to modulate these interactions and alter viral pathogenesis.

Within the hemorrhagic fever viruses, the bunyaviruses and arenaviruses have segmented genomes, three segments of negative-strand RNA in the bunyaviruses and two of ambisense RNA in the arenaviruses. This aspect of the virus biology naturally suggests a strategy for the creation of novel vaccines, whereby the understanding of which genes, and therefore which genome segments, correlate with pathogenesis, allowing the creation of mixed segment viruses as vaccine candidates (**Fig. 2**).

A candidate vaccine that has been developed against the hemorrhagic arenavirus *Lassa virus* (LASV), takes advantage of a related arenavirus, *Mopeia virus* (MOPV), which is not pathogenic in animal models, and appears not to be in human populations, although public health surveillance is limited in this case. The vaccine candidate is a reassortant containing the S RNA from LASV and the L RNA from MOPV. This virus therefore expresses the glycoprotein and nucleoprotein of LASV and is capable of inducing protective cell-mediated immunity, but without causing disease.[25,26]

This reassortant strategy is most effective if virulence maps strongly to one segment of the viral genome. If proteins or motifs correlated with virulence and the onset of disease are present on multiple

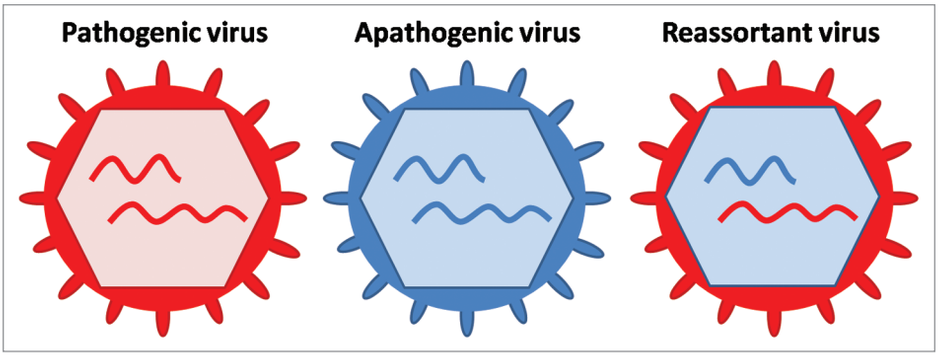

Figure 2. Reassortant multi-segment viruses. For viruses which have genomes comprised of multiple strands, 'swapping' on gene segments provides and obvious strategy for the generation of attenuated vaccine candidates, for viruses which have similar naturally occurring avirulent species or strains. By infecting cells with both viruses simultaneously, reassortant viruses can be produced and assayed for virulence.

segments, reassortant viruses may not be sufficiently attenuated to use as vaccine candidates. In the case of *Pichindé* virus, a BSL-2 model in guinea pigs for Lassa fever, reciprocal reassortants did show differences in morbidity, but neither was completely attenuated, suggesting virulence determinants across both strands of the genome.[27]

Virus-Like Particles and Pseudotyping Approaches

Virus-like particles (VLPs) are non-infectious forms of viruses that do not contain viral genetic material, and as a result cannot replicate.[28] Since VLPs contain the structural proteins of the virulent virus, they are capable of generating an effective immune response while not causing disease. As a result, they have been used both as vaccine vectors, carrying DNA of antigens of interest, as well as vaccine contributors.[29,30] A schematic of the VLP production method is shown in **Figure 3**. VLPs have been generated for a number of different viruses including influenza, Ebola and Nipah viruses,[31-33] and vaccines containing VLPs have already reached clinical trials.[34]

Another approach similar in nature is the generation of pseudo- and pseudotype viruses. Unlike true VLPs, pseudoviruses do contain nucleic material, although not that of the core virion. An example of such is that of the papillomavirus. Shi et al. developed a pseudovirus papillomavirus that encodes lymphocytic choriomeningitis virus gp33. The papillomavirus VLP core working in consort with the contained DNA successfully generated a cytotoxic T-cell response that was absent with the LCMV DNA vaccine alone.[35] Pseudotype viruses, on the other hand, consist of inert viruses (in humans) such as baculovirus with an insert of a glycoprotein gene from a virus of interest. This allows for the large-scale production of virions expressing antigens of interest in a viral vector that is non-pathogenic in humans. This approach has already been used for a number of viruses including Ebola and Rabies.[36-38]

It is clear that both of these approaches, VLPs and pseudo/pseudotype viruses, are important and promising techniques for vaccine development that will allow for effective vaccines to be produced in the future that should allow for a good immune response while keeping adverse events to a minimum.

Pathogenic and Apathogenic Viruses and Adjuvant Design

A major issue in vaccine design is vaccine safety. This is perhaps the primary consideration in the development of new vaccines. As such, the continued development of live attenuated vaccines may diminish, or become limited to more specific groups of patients, e.g., with restricted age ranges, immune status etc. A further issue with the use of live attenuated vaccines is the potential for vaccines to revert to a virulent form. The RNA-dependent RNA polymerase is highly prone to replication errors. This fact, combined with high numbers of progeny virus, affords the opportunity for the attenuating mutations to be lost. Vaccination with the live polio vaccine can result in reversion to wild-type neurovirulent poliovirus.[39] This finding has contributed to the change to the use of inactivation polio vaccine as we move closer to eradication of the disease.

In the case of adverse events, the yellow fever vaccine provides an interesting paradigm. The 17D yellow fever vaccine has been used for over 70 years, but cases of adverse events have driven the development of new, inactivated yellow fever vaccines.[40,41] A consideration of inactivated vaccines is the immunogenicity and duration of protection elicited by these vaccines. Inactivated vaccines often require repeated boosters to ensure adequate protection over time. In countries and regions with good healthcare infrastructure, this may not pose any problems. However, in other areas this is likely to be a significant drawback for a vaccination strategy.

Adjuvants are compounds which aim to enhance the immune response to vaccines. Presently, aluminum-based gels and salts are the only adjuvant licensed for use in humans. A mechanism for the effectiveness of these adjuvants has been activation of the Nalp-3 inflammasome, a critical component in innate immune function, which leads to the production of the pro-inflammatory cytokine interleukin-1β.[42] Activation of this immune pathway may drive the immune response to produce specific effectors which the vaccine alone may not.

Another potential adjuvant strategy is the use of cytokines. GM-CSF is a cytokine which leads to the production of granulocytic immune cells, including macrophages. Experimental studies

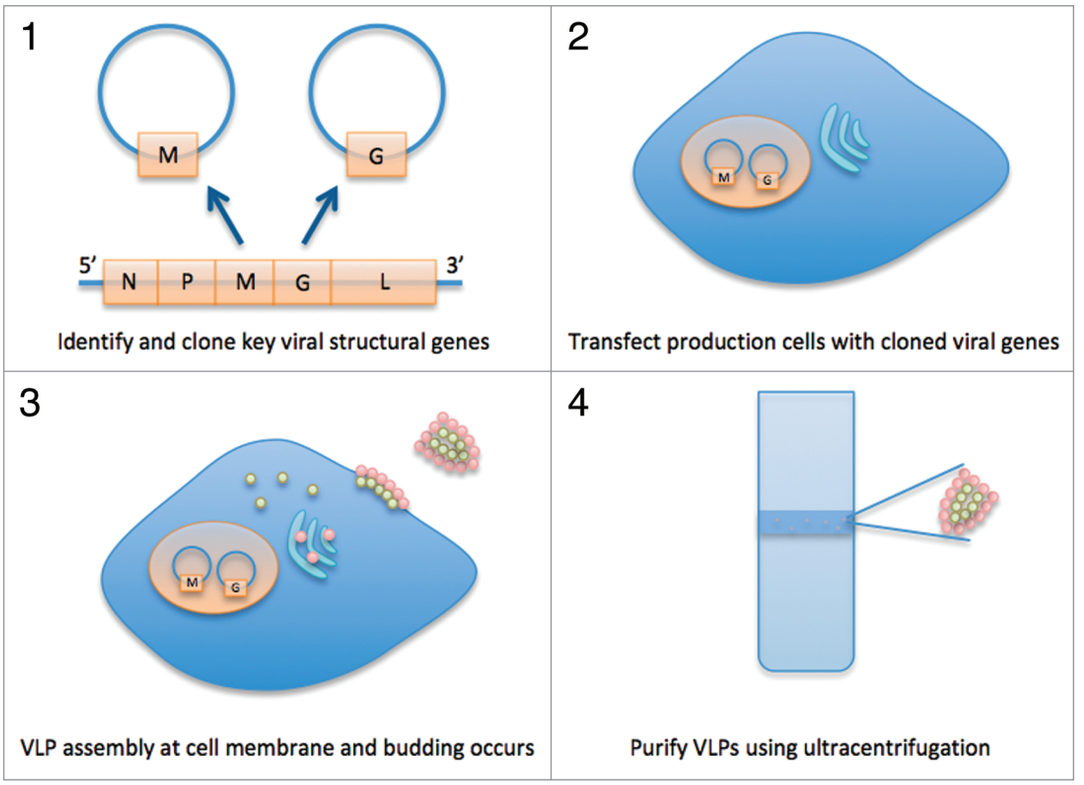

Figure 3. Virus-like particle production. Virus-like particles (VLPs) take advantage of reverse genetics systems in which the viral glycoproteins, and other viral structural proteins required to drive particle formation can be identified and cloned (1), expressed from transfected plasmids (2) and used to generate particles (3) which express the appropriate virus proteins on the surface, but contain no genetic material. These may then be purified by centrifugation (4) and used as vaccine candidates without some of the concerns which must be considered with live-attenuated viruses such as reversion to virulence.

have shown that the use of recombinant GM-CSF as an adjuvant in hepatitis B (HBV) vaccination can increase anti-HBV antibody titers significantly.[43,44] This observation suggests that using immune mediators may be a promising strategy in boosting responses to vaccination. In the context of vaccines against hemorrhagic fever viruses such as Lassa and Ebola viruses in sub-Saharan Africa, the immune status of the population must be considered. As an example, cellular responses to vaccination in HIV positive individuals may be altered due to the effects of HIV infection on immune signaling networks; stimulation of specific pathways may be required to induce appropriate responses.[45,46]

Before immunomodulatory adjuvants can be developed, it is important to understand the type of immune response required to clear infection. In one study, a recombinant mousepox virus was engineered to contain the gene for the cytokine IL-4, a B-cell growth factor. It was hypothesized that this cytokine would drive the development of a protective antibody response. However, this virus was found to be able to cause disease in mice which were genetically resistant to a virulent strain of the virus.[47] This cytokine may have 'reprogrammed' the immune response, promoting an antibody-based humoral response, but to the detriment of the cell-mediated response, the response required to provide protection against this disease. A recent study using whole transcriptome profiling of white blood cells of patients vaccinated with the yellow fever vaccine has been used to determine key immune regulators of the protective response.[48] Studies such as these may be critical in determining the immune determinants required for protection and allow the development of an adjuvant strategy which provides the 'best of both worlds': the immunogenicity and long-term protection of a live vaccine, with the safety of an inactivated vaccine. A recent study has provided an illustration of this, using antigen and toll-like receptor delivering nanoparticles to provide long-lasting protective immunity against influenza in animal models.[51]

Conclusions

In this commentary, we have discussed emerging strategies for the development of novel vaccines against emerging and biothreat viruses. The development of recombinant strategies such as pseudotyped viruses and virus-like particles is already bearing fruit in terms of new vaccine candidates progressing through the development pipeline. Recombinant and viral reverse genetics systems can also feed into this pipeline at the level of basic research, with mutated viruses being used to dissect the immune responses required for protection.

A critical point to discuss when discussing the development of vaccines against emerging and biothreat agents is the balance of immunogenicity and vaccine safety.

A vaccine that is designed to be administered to a large population to protect against a disease which may have only a small risk of serious disease must have a very low risk of adverse events, but in the case of an epidemic of a highly lethal disease such as Zaire Ebolavirus, is the use of a vaccine with a higher risk of adverse events justified?

It is also important to consider what type of immunity is required. For childhood vaccinations and global disease eradication efforts, a vaccine which provides long-term protection, ideally without the requirement for multiple boosters would be ideal. However, in the case of local responses to epidemics and ring vaccination efforts, the long-term protective effect is secondary to ensuring a minimal risk of vaccine-associated adverse events and a rapid, strong immune response.

Vaccination remains the most effective way to control viral diseases. For emerging and biothreat agents, the vaccine landscape is very different, given the hope that either vaccines against these diseases will never have to be used, or given the economic concerns of balancing the cost of developing and licensing a vaccine, versus the ability of the vaccine to recoup that expense. The development of novel approaches to vaccine development, beyond existing methods of attenuation by repeated passage or whole pathogen inactivation, may alleviate many of the concerns associated with vaccine safety, as we are able to move away from empirically attenuated vaccines, for which the mechanisms behind attenuation, and in the development of adverse effects, are unknown. These approaches will continue to generate novel vaccine candidates which show promise in the prevention and, in some cases, treatment of emerging viruses. As our understanding of the mechanisms of immune protection at the molecular level become better defined for specific pathogens, our ability to target specific immune effector arms, that are known to be required for clearance of the wild-type pathogen or for the establishment of a long-lasting immunity, may allow us to develop the next generation of vaccines.

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